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# The use of delta-tocotrienol and lovastatin for anti-osteoporotic therapy

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ABSTRACT

*Aims:* Statins are competitive inhibitors of HMGCoA reductase and are commonly used as anti-hypercholesterolemic agents. Experimental studies clearly demonstrate the beneficial effects of statins on bone. Tocotrienols have also been shown to have anti-osteoporotic effects on the skeletal system. This study was conducted to observe the effect of a combination of delta-tocotrienol and lovastatin on structural bone histomorphometry and bone biomechanical strength in a postmenopausal rat model at clinically tolerable doses, and to compare it with the effect of delta-tocotrienol or lovastatin.
*Main methods:* Forty-eight female Sprague Dawley rats were randomly divided into six groups: baseline control; sham-operated control; ovariectomised control; ovariectomised + 11 mg/kg lovastatin; ovariectomised + 60 mg/kg delta-tocotrienol and ovariectomised + 60 mg/kg delta-tocotrienol + 11 mg/kg lovastatin. These treatments were given via oral gavage daily for eight weeks. After sacrificing the rats, the left and right femurs were dissected and processed for bone histomorphometric analysis and a bone biomechanical test, respectively.
*Key findings:* Delta-tocotrienol in combination with lovastatin significantly improved the trabecular volume, trabecular number, trabecular thickness and trabecular separation; and it significantly increased bone strength in oestrogen-deficient rats. Delta-tocotrienol alone enhanced bone formation and maintained bone strength in ovariectomised rats. Delta-tocotrienol plus lovastatin treatment promoted better trabecular volume and trabecular number and received higher load than delta-tocotrienol alone. Lovastatin alone was not effective.
*Significance:* Thus, the combination of delta-tocotrienol and lovastatin has the potential to be used for anti-osteoporotic therapy in postmenopausal women.



## Introduction

Osteoporosis is a costly global health problem [16]. It is a metabolic disorder associated with low bone density and microarchitectural impairment leading to fractures [35]. Osteoporosis is more prevalent in postmenopausal women because oestrogen deficiency suppresses osteoclast apoptosis and induces osteoblast apoptosis, resulting in net bone loss [6,31,36]. Regardless of the effectiveness of the current treatments, identification of a new agent which is effective, safe and well tolerated is mandatory.

Statins are commonly prescribed lipid-lowering agents, which have been shown to exhibit favourable effects on bone in high oral doses but not in lipid lowering doses in experimental studies [2,17]. The biological effects of statins are expressed via the inhibition of mevalonate pathway [23]. Postmenopausal women are susceptible to both cardiovascular diseases and osteoporosis [40]. However, a high dose of statins could lead to undesirable toxicity effects, such as hepatotoxicity, muscle toxicity and rhabdomyolysis [10,14]. Thus, postmenopausal women cannot benefit from statin bone anabolic and lipid lowering effects safely at the same time.

Tocopherols and tocotrienols are vitamin E isomers. Annatto bean vitamin E consists of 100% tocotrienols (10% gamma-tocotrienol and 90% delta-tocotrienol), thus providing a convenient source of delta-tocotrienol [15]. Previous studies have shown that 60 mg/kg/day of palm oil tocotrienols prevents and even reverses bone loss in various osteoporotic-animal models of osteoporosis [2,3]. Tocotrienols are superior to tocopherols in their effect on bone metabolism [30]. It has been reported that the anti-osteoporotic effect of tocotrienols is exerted through the inhibition of mevalonate pathway [7].

Since both statins and tocotrienols suppress mevalonate pathway, giving delta-tocotrienol in combination with lovastatin may have synergistic or additive beneficial effects on bone health. Moreover, the bone-beneficial effects of statins can be achieved at clinically tolerable doses with the co-administration of delta-tocotrienol.

Our previous study investigated the effects of the combined treatment on osteoblast and osteoclast activity in ovariectomised rats [1]. The results were promising. Therefore, this study was intended to continue the investigation of the effect of delta-tocotrienol in combination with lovastatin on bone structure and strength, and to compare it

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with the effect of delta-tocotrienol or lovastatin in oestrogen-deficient ovariectomised rats. The results from the current research may contribute to the development of a new therapy to prevent and treat osteoporosis.

## Materials and methods

### *Experimental animals*

Forty-eight female Sprague–Dawley rats, aged three months, weighing between 200 and 250 g were used in this study. Rats were received from the Laboratory Animal Resource Unit, Faculty of Medicine, Universiti Kebangsaan Malaysia. Two rats were kept in a cage under 12-hour natural light–dark cycles. They were provided with commercial food pellets (Gold Coin, Selangor, Malaysia) and tap water ad libitum.

### *Experimental design*

Following a week of acclimatization, the rats were grouped randomly into six groups of eight rats. Group one was the baseline control (BC) which was sacrificed at the onset of the study. Group two was the sham-operated group (SHAM), which was given olive oil and 0.5% carboxymethylcellulose as vehicles. Groups three, four, five and six were ovariectomised. Group three was the ovariectomised control (OVXC) group, which was given olive oil and 0.5% carboxymethylcellulose as vehicles. Group four was treated with 11 mg/kg/day lovastatin and an equivalent volume of olive oil as vehicle (OVX + LOV). Group five was treated with delta-tocotrienol 60 mg/kg/day and an equivalent volume of 0.5% carboxymethylcellulose as vehicle (OVX + TT). Group six was treated with delta-tocotrienol 60 mg/kg/day and lovastatin 11 mg/kg/day (OVX + LOV + TT).

Ovariectomy was performed according to Estai et al. [12]. Prior to ovariectomy, the rats were weighed and anaesthetized with a combination of ketamine 75 mg/kg rat body weight and xylazil 10 mg/kg rat body weight, mixed in a ratio of 1:1 and given intramuscularly as 0.1 ml/100 g. Following anaesthesia, the rats were placed in a supine position and the fur of the abdomen was shaved and removed completely with an electrical clipper. The skin was washed and disinfected with ethanol 70% and povidone iodine, respectively. Bilateral ovariectomy was performed on the rats using the ventral approach. A midline abdominal incision was made using a sterilized scalpel blade, approximately halfway between the middle of the back and the base of the tail. Sterilized scissors and forceps were used to gain access to the peritoneal cavity. After gaining access to the peritoneal cavity, both ovaries were identified. The blood vessels were ligated to prevent bleeding. The connection between the uterine horn and fallopian tube was cut, and the ovaries were exteriorized by gentle retraction and moved out. Following the removal of the ovaries, both the peritoneal cavity and the skin were closed aseptically using absorbable sutures. After operation, the rats were placed under electrical light to prevent hypothermia. For sham operation, the ovaries were exposed as above and gently manipulated but not excised and left in-situ. Daily dressing with povidone iodine and intramuscular injection of baytril 5% at a dose of 10 mg/kg rat body weight was given daily for 7 days.

Following eight weeks of treatment, the rats were sacrificed humanely with diethyl ether 1 day after the last treatment. The left femurs were taken for bone histomorphometry and the right femurs for biomechanical strength test. The current study was approved by Universiti Kebangsaan Malaysia Research and Animal Ethics Committee, certificate number: (PP/FAR/2011/IMA/27-JANUARY/352-JANUARY-2011–DECEMBER-2012).

### *Preparation of treatment*

Annatto tocotrienols containing 10% gamma tocotrienol and 90% delta-tocotrienol (American River Nutrition, Hadley, USA) were diluted in olive oil (Bertolli Classico, Italy) and given daily via oral gavage at a dose of 60 mg/kg/day for 8 weeks [1,38].

Mevacor tablets containing 40 mg of lovastatin were grounded and suspended in 0.5% carboxymethylcellulose (Sigma-Aldrich, St. Louis, USA) solution and administrated daily via oral gavage at a dose of 11 mg/kg/day for 8 weeks [1,17].

### *Bone histomorphometry*

The distal part of the left femur was cleaned from the tissues, fixed in 70% alcohol and divided sagittally into 2 fractions using a bone cutting saw (Black & Decker Rotary Tools, USA). One fraction of the left femur was used to measure the structural histomorphometric indices. According to Difford [8], the undecalcified portion of the left femora were infiltrated and embedded in polymer methyl methacrylate (Osteo-Bed Bone Embedding Kit; Polysciences, USA). Following the polymerization process, the resin blocks were sectioned at 8 μm thickness by a microtome (Leica, Wetzlar, Germany) and stained with Von Kossa stain. Structural parameters were examined by utilizing a Nikon Eclipse 80i microscope (Nikon Instrument Inc., US) with an image analyzer software Pro-Plus v.5.0 (Media Cybernetics, Silver Spring, MD, USA). Structural bone histomorphometric parameters were expressed according to the American Society of Bone Mineral Research Histomorphometry Nomenclature Committee [34]. All the structural histomorphometric analyses were performed at the metaphyseal region, which was located 3 to 7 mm from the lowest point of the growth plate and 1 mm from the lateral cortex, excluding the endocortical region. This is the secondary spongiosa area, which is rich in the newly formed trabecular bone. Bone volume/tissue volume (BV/TV %), trabecular thickness (Tb.Th μm), trabecular number (Tb.N $mm^{-1}$) and trabecular separation (Tb.Sp μm) were the structural histomorphometric indices.

### *Bone biomechanical test*

The right femora were cleaned of soft tissues and kept wet by wrapping them with gauze dipped in phosphate-buffered saline and rewrapped with aluminium foil. The biomechanical properties of the femora were evaluated using an Instron Universal Testing Machine (5560 Instron, Canton, MA, USA) and Bluehill 2 software package (Instron, Canton, MA, USA). Three point bending configuration of the right femur of all the rats was performed to assess the bone biomechanical properties, in which the right femur was mounted on two inferior supports and the load was applied to the mid-diaphysis on the anterior surface, such that the anterior surface was in compression and the posterior surface in tension. A loading speed of 5 mm/min and a span length of 10 mm were applied until the femur was fractured (Fig. 1). The Bluehill software was utilized to transcribe the load N, strain MPa and stress mm/mm parameters. The Young's modulus MPa was represented by the slope value of the stress–strain curve.

### *Statistical analysis*

Data analysis was performed using the Statistical Package for Social Sciences software (Version 19, SPSS, Chicago, IL, USA). The Kolmogorov–Smirnov test was used as a normality test. Normally distributed variables were analyzed by using analysis of variance (ANOVA) followed by Tukey's post-hoc test. The statistical differences were assumed significant at $P < 0.05$. The results were expressed as mean values ± SEM.

## Results

### *Bone structural histomorphometry*

At the end of the study, BV/TV %, Tb.Th μm, Tb.N $mm^{-1}$ and Tb.Sp μm of the OVXC group were significantly reduced as compared to the BC

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